

DOCKET NO.: ALLE0042-100 (17007 CON)
U.S. Serial No. 09/845,514

PATENT

REMARKS

Upon entry of this response, claims 1, 8, 9, 17, 24, 25, and 28-31 will be pending. Claims 1, 17 and 30-31 have been amended, and claims 2-7, 18-23 and 32-33 have been canceled without prejudice. Claims 1, 17 and 30 have been amended to include subject matter from previous (canceled) claims 2, 5, 18 and 21. For example, each of the present claims recite specific combinations of botulinum toxins (that is, a combination of botulinum toxin type A and type B, or a combination of botulinum toxin type A and type E) that are present in a single composition or are administered simultaneously to a patient. Thus, the amendments to the claims are fully supported by the specification as originally filed. No new matter is added.

Claims 1-9, 17-25 and 28-33 are rejected under 35 U.S.C. §103(a) as allegedly being obvious over the Ludlow et al. (1992), in view of Simpson (1991) and further in view of Jankovic et al. (1991). Applicant respectfully disagrees.

As an overview, the claimed invention is not obvious because:

(I) Prior art **does not teach a combined** (i.e., sequential or simultaneous administration) use of BoNT-A and non-type-A-BoNT

(II) Prior art **teaches away** from a simultaneous administration of a BoNT-A and a non-type-A-BoNT

(III) Dr. Mitchell Brin Declared in a declaration that at the filing date of the present invention, the use of two or more types of botulinum toxin together would be considered **foolhardy and dangerous**.

The claimed invention is directed to a simultaneous (e.g., combined) administration of a botulinum toxin type A ("BoNT-A") and a non-type A botulinum toxin ("non-type-A-BoNT", e.g., botulinum toxin types B, C, D, E, F and/or G). The claimed invention is not obvious for at least the following reasons.

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(I) Prior Art Does Not Teach Combined (i.e., Sequential or Simultaneous Administration) Use of BoNT-A and Other-BoNT

First, it is important to understand that a “combined” use is where each of the botulinum toxin type actually exerts a therapeutic effect concurrently with other botulinum toxins being administered. Thus, an administration of a second type of botulinum toxin after the first is no longer effective (e.g., due to immunity) is not a “combined” use, but simply a replacement of one botulinum toxin type with another. Combined use is not the same as replacement use.

Further, it is important to recognize that the combined use of different toxin types, sequentially or simultaneously (as recited by the pending claims), was not even known prior to the filing date of the claimed invention.

With regard to Ludlow et al. specifically, the reference actually teaches the use of botulinum toxin type F solely as a **replacement** for type A, not combined with BoNT-A in any way. Specifically, Ludlow et al. discloses that the patients being treated

- had antibodies to BoNT-A (rendering them immune/unresponsive to treatment with BoNT-A),
- had impaired movements, i.e., torticollis and oromandibular dystonia¹,
- were treated with botulinum toxin type F and were relieved from the impaired movements.²

Clearly, Ludlow et al. only teaches that botulinum toxin type F can be a replacement for BoNT-A when type A is no longer effective due to antibody against type A. Moreover, Ludlow et al. is a scientific article reporting experimental finding that botulinum toxin type F is effective in treating a neuromuscular disorder. Accordingly, Ludlow et al. must

¹ Ludlow et al. at page 350, first column, discloses that “[b]efore the toxin was injected, the four patients were videotaped during impaired movements due to torticollis and during conversational speech affected by oromandibular dystonia and stuttering.”

² Ludlow et al. at 349, second column, and at 350, first column.

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have followed an experimental protocol that proves that botulinum toxin type F is effective for treating a neuromuscular disorder, and that botulinum toxin type F is the sole agent responsible for the treatment of the neuromuscular disorder, not BoNT-A. Such protocol would **exclude**, for example, the administration of BoNT-A because the administration of type A along with type F would make it unclear as to whether and how much of the treatment is due to botulinum toxin type F as opposed to type A. Further, there is no reason for administering BoNT-A to the patients in Ludlow et al., because these patients are unresponsive to BoNT-A to begin with. Thus, the combined use of BoNT-A with a non-type-A-BoNT was not known with respect to Ludlow et al. Ludlow et al. only teaches that botulinum toxin type F can be used as a replacement for BoNT-A.

(II) Prior Art Teaches Away From a Simultaneous Administration of a BoNT-A and a Non-Type-A-BoNT

Clearly, it was *not* known that BoNT-A can be administered with a non-type-A-BoNT, sequentially or simultaneously. Further, one of ordinary skill would not be motivated by Ludlow et al. to administer BoNT-A in combination with another toxin type, sequentially or simultaneously. As discussed above, Ludlow et al. relates to a patient population who is unresponsive to BoNT-A. Thus, there would be no reason, and no motivation, for administering BoNT-A to these patients. Moreover, Simpson and/or Jankovic et al. would not cure the deficiency of Ludlow et al. as they are silent with respect to the administration of more than one type of botulinum toxin, where each type of botulinum toxin concurrently exerts a therapeutic effect.

Assuming, *arguendo*, that Ludlow et al. teaches the sequential administration of BoNT-A and then botulinum toxin type F, the Office Action's allegation that one of skill in the art would have been motivated to simultaneously administer BoNT-A and non-type-A-BoNT is wrong. For example, at the time when the present patent application was filed, **one of ordinary skill would believe that the administration of BoNT-A at the same time with another toxin type would result in an overall decrease**

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effectiveness (e.g., decrease therapeutic duration) for treating a neuromuscular disorder. Thus, the prior art actually teaches away from simultaneous administration of BoNT-A and another toxin type.

Simpson "describes that all types of botulinum toxin depress neurogenic release of acetylcholine." Simpson also discloses that the mechanism of action of botulinum toxin comprises three steps: binding, translocation and lytic.³ Moreover, it was well known that BoNT-A has the longest lasting therapeutic effect among the toxin types. For example, Ludlow et al. teaches that BoNT-A has a longer therapeutic effect than that of type F.⁴

One of ordinary skill would not be motivated to simultaneously administer BoNT-A with a non-type-A-BoNT because these toxins would **competitively compete** at the binding, translocation and lytic step. Further, one of ordinary skill would believe that this competition would decrease the entry of BoNT-A into cells in favor of another toxin type. The decreased entry of BoNT-A into cells would mean that the contributing effect of BoNT-A would be less. If the contributing effect of BoNT-A decreases, then one of ordinary skill would expect that the overall therapeutic duration would also decrease, as BoNT-A has the longest lasting therapeutic effect. In fact, this concern was still relevant as of 2001, more than 7 years after the priority filing date (June 10, 1993) of the claimed invention. For example, Adler et al. (Toxicon, 2001, 39:233-243, hereinafter "the Adler reference", Exhibit 1) discloses at page 240, second column, that

[w]ith simultaneous application of serotypes A and E, competition for a common receptor could result in a reduced binding of each serotype...Under this condition, recovery from paralysis may exhibit a time course that is no longer dominated by BoNT/A. In addition, simultaneous injection of BoNT/A and BoNT/E may also lead to

³ See Simpson at pages 169-170.

⁴ Simpson at page 350, last paragraph.

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competition for internalization sites as well as for access to substrate.

Thus, Ludlow et al. and Simpson actually teach away from the administration of BoNT-A with another type of botulinum toxin. In fact, it is the Applicant who surprisingly discovered that BoNT-A may be administered with a non-type-A-BoNT without compromising the therapeutic duration of type A.

After the filing date of the claimed invention, the scientific community published data that supports the claimed invention. For example, the Adler reference provides experimental data supporting the fact that the therapeutic duration of BoNT-A would not be compromised by the simultaneous administration of another botulinum toxin. For example, the Adler reference, in the abstract, states:

... BoNT intoxication is lengthened by exposure to serotype A, but not shortened by exposure to serotype E, the duration of BoNT/A intoxication appears to be determined predominantly by the intracellular stability of catalytically active BoNT/A light chain.

In brief, after the priority date of the claimed invention, the Adler reference provides data proving that the therapeutic duration of BoNT-A is due to its long biological half life. Since BoNT-A has a longer biological half life than the other types, it will be available to act on the cell to decrease acetylcholine release, even after when the other administered toxins have been degraded by the cell. Thus, the Adler reference provides experimental data supporting Applicant's discovery that the therapeutic duration of BoNT-A would not be compromised by the simultaneous administration of another botulinum toxin.

Further, the Pearce et al. (U.S. Patent No. 6,087,327) supports the surprising finding by Applicant that the administration of a non-type-A-BoNT simultaneously with a BoNT-A does not compromise the therapeutic effectiveness (e.g., duration) of BoNT-A. For example, Example 3 and Figure 5 of Pearce et al. demonstrate the unexpected results that the duration of effect of the simultaneously administered type A and type B

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does not have a duration of effect that is shorter than that of type A alone. That is, the data show that a simultaneous administration of a non-type-A-BoNT did not compete and compromise/shorten the duration of effect of type A. Example 3 and Figure 5 surprisingly show that the duration of effect of type A and type B administered together was at least as long as type A.

In addition to the data surprisingly showing that the non-type-A-BoNT does not compromise the duration of type A, the data also shows that the longer lasting effect was synergistic, i.e., more than simply the additive duration of type A and type B. For example, type A, type B and types A/B combination achieved 50% paralytic effects for about 7.5 days, about 6.5 days and about 15.5 days, respectively. The duration of 15.5 days is longer than the additive duration of 14 days (7.5 plus 6.5 days) expected from the effects of types A and B alone.

In brief, the claimed invention is not obvious because the administration of BoNT-A at the same time with another toxin type does not result in an overall decrease effectiveness (e.g., decrease therapeutic duration), as one of ordinary skill would expect from the teaching of the prior art.

(III) The Use of Two Or More Types of Botulinum Toxin Together Would Be Considered Foolhardy And Dangerous.

As asserted in the previous responses, Dr. Mitchell Brin Declared in a declaration that at the filing date of the present invention, the use of two or more types of botulinum toxin together would be considered **foolhardy and dangerous**. The Brin declaration is clear and substantial evidence demonstrating that a person of ordinary skill in the art would not be motivated to combine two or more different types of botulinum toxin and simultaneously administer the combination of botulinum toxins to treat a patient.

Applicant respectfully reminds the Office that the Office must give deference and weight to evidence presented in a declaration, i.e. the Brin declaration.

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In view of the foregoing, Applicant submits that the pending claims are in condition for allowance, and an early Office Action to that effect is earnestly solicited.

Respectfully submitted,



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Doc No. 2441720



Toxicon 39 (2001) 233–243

Toxicon

www.elsevier.com/locate/toxicon

Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle^{*}

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Received 5 May 1999; accepted 6 March 2000

Abstract

Botulinum neurotoxin serotypes A (BoNT/A) and E (BoNT/E) inhibit neurotransmitter release from peripheral cholinergic nerve terminals by cleaving different sites on SNAP-25, a protein involved in synaptic vesicle docking and exocytosis. Since recovery from BoNT/A is protracted, but reversal of BoNT/E intoxication is relatively rapid, it was of interest to determine whether sequential exposure to BoNT/A and BoNT/E could provide insight into the factors responsible for persistence of BoNT action. Extensor digitorum longus (EDL) muscles from rats were injected locally with 5 mouse LD₅₀ units of BoNT/A or 20 mouse LD₅₀ units of BoNT/E; these doses were selected to produce total paralysis of EDL muscles within 48 hr. Additional groups of rats were injected sequentially with either BoNT/A followed 48 h later by BoNT/E or with BoNT/E followed 48 h later by BoNT/A. Muscle tensions were elicited in situ in response to supramaximal stimulation of the peroneal nerve to monitor recovery from BoNT intoxication. Tensions returned to 53% and 94% of control, respectively, 7 and 15 days after injection of BoNT/E. In contrast, tensions in muscles injected with BoNT/A returned to only 2% and 12% of control at these time points. Preparations injected sequentially with BoNT/A followed by BoNT/E or with BoNT/E followed by BoNT/A exhibited slow recovery times resembling those recorded in the presence of BoNT/A alone. Pronounced atrophy of the EDL muscle was observed in rats injected with BoNT/A or in those receiving serotype combinations in either sequence, whereas no loss of muscle mass was observed in animals treated with BoNT/E alone. Data suggesting that BoNT/E can enter BoNT/A-treated preparations was obtained by findings that 3,4-diaminopyridine, which readily reversed muscle paralysis after BoNT/A exposure, lost this ability within 1 h of BoNT/E addition. Evidence that BoNT/E was able to cleave SNAP-25 at its characteristic site during sequential neurotoxin exposure was demonstrated by western blot analysis of cultured primary cortical neurons. Since the sequential exposure studies indicate that recovery from BoNT intoxication is lengthened by exposure to serotype A, but not shortened by exposure to serotype E, the duration of BoNT/A intoxication appears to be determined predominantly by the intracellular stability of catalytically active BoNT/A light chain. Published by Elsevier Science Ltd.

Keywords: Botulinum neurotoxin; Persistence; Rat; Muscle tension; Cortical neurons

^{*} Opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996.

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1. Introduction

The seven distinct serotypes (A-G) of *Clostridium botulinum* neurotoxins (BoNTs) block acetylcholine release from peripheral cholinergic nerve terminals resulting in flaccid paralysis (Habermann and Dreyer, 1986; Simpson, 1989). The specific target for BoNT/A and BoNT/E is the 25 kDa vesicle docking protein, SNAP-25; BoNT/A cleaves the last 9 residues from the C-terminus, whereas BoNT/E cleaves a larger 26 residue fragment from the same region of this protein (Binz et al., 1994; Valdivyathan et al., 1999). The enzymatically active portion of the ~150 kDa BoNT is the ~50 kDa light chain; the role of the ~100 kDa heavy chain resides in binding to cholinergic nerve endings and in promoting intracellular penetration via receptor mediated endocytosis (Simpson, 1989; Montecucco et al., 1994; Schiavo et al., 1995).

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes (Erdal et al., 1995). Intramuscularly injected BoNT/C, which in human muscle has an effective duration similar to that of BoNT/A, appears to be the only exception (Eleopra et al., 1997). The remarkable persistence of BoNT/A action has led to its widespread use in the treatment of disorders of muscle tone and movement (Scott, 1989; Shantz and Johnson, 1992; Jankovic and Brin, 1997). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A would also make intoxication by this serotype difficult to treat, particularly in a combat or terrorist environment (Franz et al., 1997).

The basis for the differences in serotype persistence is unknown. One possibility is that BoNT/A remains enzymatically active for longer periods in the nerve terminal than BoNT/E (Keller et al., 1999). It is also possible that the truncated SNAP-25 fragments resulting from BoNT/A and BoNT/E action are inherently inhibitory, and that the fragment generated by BoNT/A remains in the nerve terminal for longer periods (Eleopra et al., 1998; Raciborska and Charlton, 1999). In the case of BoNT/A, cleavage of SNAP-25 results in a 9-mer C-terminal peptide (P9) and a 197-mer truncated SNAP-25 fragment (P197). BoNT/E generates a 26-mer peptide (P26) and a 180-mer truncated SNAP-25 fragment (P180) (Binz et al., 1994). Inhibition of exocytosis by both small and large SNAP-25 fragments has in fact been demonstrated (Gutierrez et al., 1995, 1997; Ferrer-Montiel et al., 1998; Huang et al., 1998); however, there is no direct evidence that elimination of SNAP-25 fragments underlies recovery from BoNT intoxication.

To determine whether SNAP-25 cleavage products are responsible for the duration of BoNT action or whether persistence of intoxication reflects continued

enzymatic activity of internalized toxin, information on the elimination rate of P197 and P180 or direct measurement of the residence time of BoNT light chain would be desirable. Detection of intracellular BoNT is difficult, however, due to the low levels of toxin associated with inhibition of transmitter release (Erdal et al., 1995), and degradation rates for SNAP-25 fragments have not been determined. To circumvent these limitations, an indirect approach was adopted in the present study. The strategy was based on monitoring alterations in muscle tension in situ following injection of BoNT/A and BoNT/E individually or consecutively in the rat extensor digitorum longus (EDL) muscle. The study was designed to reveal whether the duration of BoNT-mediated paralysis was determined principally by persistence of active toxin or, alternatively, by the lifetime of truncated SNAP-25 fragments.

The hypothesis was formulated as follows. If both serotypes have a brief lifetime in the nerve terminal, and the longer paralysis time observed with BoNT/A is due to a greater persistence of P197 relative to P180, exposure of muscles to BoNT/E after BoNT/A would convert P197 to P180 (Lawrence et al., 1997) and accelerate recovery (Eleopra et al., 1998). However, if BoNT/E is given first, a subsequent injection of BoNT/A should have no effect on recovery time, since the cleavage site for BoNT/A has already been removed by prior exposure to BoNT/E (Fig. 1). Hence, recovery of tension in muscles paralyzed initially by BoNT/E should not be prolonged by subsequent exposure to BoNT/A. Alternatively, if recovery from paralysis is governed by the lifetime of active toxin, and the stability of intracellular BoNT/A exceeds that of BoNT/E, recovery times should be determined by BoNT/A regardless of whether it is added before or after BoNT/E. Our results are consistent with the latter hypothesis, although evidence in support of the former proposal has recently been published in human muscle exposed simultaneously to BoNT/A and BoNT/E (Eleopra et al., 1998). The basis for this disparity is unknown but may reflect differences in the species studied or in the method of toxin administration.

2. Materials and methods

2.1. In situ contraction of EDL muscle

In situ tension measurements were performed on male CD rats with initial weights of 220-290 g. The animals were maintained under an approved American Association for the Accreditation of Laboratory Animal Care (AAALAC) program as described previously (Adler et al., 1992). The left EDL muscle was injected

i.m. with 5 mouse LD₅₀ units (MU) of BoNT/A in a volume of 15 µl or 20 MU of BoNT/E in 20 µl under sodium pentobarbital anesthesia (50 mg/kg, i.p.). These doses produced near total paralysis of the injected muscle within 24 h and total paralysis by 48 h with no apparent systemic toxicity. The relative potencies of BoNT/A and BoNT/E on rat EDL muscle were similar to those reported by Sellin et al. (1983) and Adler et al. (1996). Paralysis was indicated by the absence of a reflex toe spread in the injected limb and confirmed by electrophysiological recordings. In rats receiving both BoNT/A and BoNT/E, an interval of 48 h was maintained between the two injections to allow complete proteolysis of SNAP-25 by the first serotype (Binz et al., 1994; Schiavo et al., 1995; Valdyanathan et al., 1999).

Muscle contractions were recorded from rats anesthetized with chloral hydrate (400 mg/kg, i.p.) at 3, 7, 15 and 30 days after BoNT injection by standard procedures (Sellin et al., 1983; Adler et al., 1992, 1996). The peroneal nerve was stimulated for 0.2 ms via bipolar platinum electrodes using a Grass S88 stimulator (Watwick, RI, USA) at voltages sufficient to produce supramaximal tension. Responses were measured using Grass isometric force transducers and

recorded on a Gould WindoGraf chart recorder (Valley View, OH, USA).

2.2. In vitro tension measurements

In vitro studies were performed on diaphragm muscles removed from male CD-1 mice to test the sensitivity of BoNT-treated muscles to 3,4-diaminopyridine (3,4-DAP); this potassium channel blocker has been shown to antagonize the actions of BoNT/A selectively, having little or no effect on muscles exposed to other BoNT serotypes (Sellin et al., 1983; Simpson, 1986; Adler et al., 1996). Mice were sacrificed by decapitation after being rendered unconscious by excess CO₂. Hemidiaphragms with attached phrenic nerves were dissected and mounted in tissue baths containing physiological solution of the following composition (mM): NaCl, 135.0; KCl, 5.0; MgCl₂, 1.0; CaCl₂, 2.0; NaHCO₃, 15.0; NaHPO₄, 1.0; glucose, 11.0; pH 7.3. The solution was maintained at 37°C and bubbled with a gas mixture of 95% O₂/5% CO₂. The phrenic nerve was stimulated supramaximally with 0.2-ms pulses at 0.1 Hz; the resulting muscle twitches were measured with isometric force transducers (model FORT 10, WPI, Sarasota, FL, USA) and analyzed with pClamp software (Axon Instruments, Foster City,

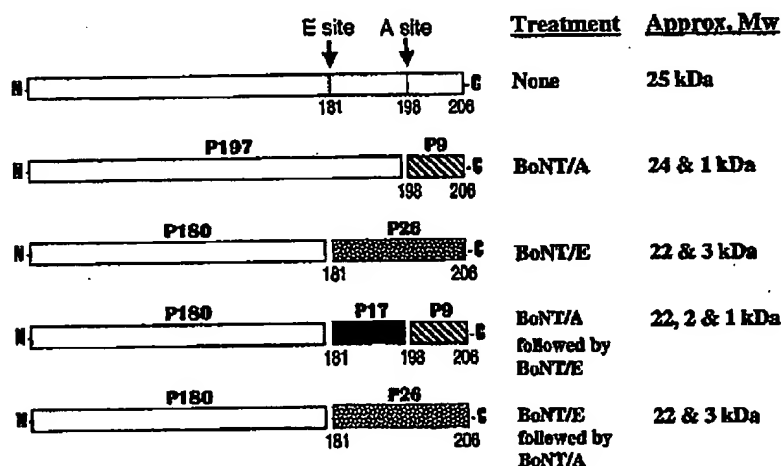


Fig. 1. Schematic diagram showing the cleavage products formed from individual and combined action of BoNT/A and BoNT/E. BoNT/A cleavage occurs between Gln¹⁹⁷ and Arg¹⁹⁸ and yields a 197-mer truncated SNAP-25 fragment (P197) that remains attached to the active-zone membrane and a 9-mer peptide that is released into the cytoplasm (P9). Cleavage by BoNT/E occurs between Arg¹⁸⁰ and Ile¹⁸¹ and results in a membrane-anchored 180-mer truncated SNAP-25 fragment (P180) and a 26-mer peptide that is released into the cytoplasm (P26). Sequential injection of BoNT/A and BoNT/E yields three SNAP-25 fragments; one fragment (P9) is generated from the initial action of BoNT/A on SNAP-25 and the others (P180) and a 17-mer peptide (P17) arise from the action of BoNT/E on P197. Sequential administration of BoNT/E followed by BoNT/A gives rise to the same cleavage products as does exposure to BoNT/E alone.

CA, USA). BoNT/A and BoNT/E were added from concentrated stock solutions stored at -80°C .

2.3. Tissue culture and protein isolation

Cortical cells obtained from 16- to 18-day-old embryonic rat pups were maintained on a monolayer of glial cells at 37°C in a humidified atmosphere of 90% air/10% CO_2 for 6 weeks. Growth medium consisted of Eagle's minimum essential medium supplemented with 5% heat-inactivated horse serum and a mixture of complex factors (Keller et al., 1999). Cortical cells were used in western blot assays to monitor the toxin-induced cleavage of SNAP-25. 50 pM BoNT/A or 90 pM BoNT/E were applied individually or sequentially to cells. For the sequential application, BoNT/A was added first, followed 24 h later by BoNT/E. For both individual or sequential incubations, cells were harvested 48 h after the first toxin exposure with the aid of 0.1% trypsin in Hanks balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) for 3 min followed by centrifugation at 1500 rpm for 1 min. Cells were solubilized in 1% sodium dodecyl sulfate and stored at -20°C . Protein concentration was measured by the BCA method using bovine serum albumin as the external standard (Pierce Chemical, Rockford, IL, USA).

2.4. Gel electrophoresis and western transfer

All buffer reagents were obtained from BioRad (Hercules, CA, USA). Gel electrophoresis was performed by the method of Laemmli (1970) using separating gels of 16.5% acrylamide (16.5% T, 2.5% C) with a 4.5% acrylamide stacking gel. Running buffer was 0.1 M Tris-tricine, pH 8.3 (Schagger and von Jagow, 1987). Protein dissolved in SDS was mixed with an equal volume of 5% β -mercaptoethanol, 20% glycerol and 0.05% bromophenol blue and separated by electrophoresis. Antibodies were diluted 1000-fold in blocking solution (5% Carnation non-fat dry milk in 20 mM Tris and 0.5 M NaCl, pH 7.5) and incubated with the membrane for 2 h at 30°C with gentle agitation.

Primary antibodies were the anti-syntaxin clone HPC-1 from Sigma-Aldrich and SMI-81 from Sternberger Monoclonals (Lutherville, MD, USA); SMI-81 recognizes the N-terminal region of SNAP-25. Goat anti-mouse conjugated to alkaline phosphatase was used to visualize primary immuno-complexes using the substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich).

2.5. Botulinum neurotoxin preparations

BoNT/A and BoNT/E complexes were obtained

from Wako BioProducts (Richmond, VA, USA.) at concentrations of 1 mg/ml with reported toxicities of 2×10^7 and 1×10^7 MU per mg, respectively. BoNT/E was activated by limited proteolysis at 37°C for 30 min with bovine pancreas trypsin type XI (0.3 mg/ml) in 30 mM HEPES at pH 6.8, followed by addition of soybean trypsin inhibitor type I-S (0.5 mg/ml) for 15 min (Molgo et al., 1989). Stock solutions (100 nM) of each neurotoxin were prepared by dilution with 200 mM NaCl, 50 mM Na acetate, 0.1% gelatin, pH 6.0, aliquoted and frozen at -80°C until use.

For injections of rat EDL muscle, stock solutions were diluted with 0.9% saline containing 0.1% gelatin to yield working concentrations of 330 MU/ml for BoNT/A and 1000 MU/ml for BoNT/E. For the in vitro mouse diaphragm preparations, appropriate volumes of the 100 nM stock solutions were mixed with physiologic solution to yield 300 pM BoNT/A and 500 pM BoNT/E; these concentrations produced complete muscle paralysis within 90 min. For rat cortical cells, toxin was added to the growth medium at a final concentration of 50 pM for BoNT/A and 90 pM for BoNT/E. For all components of the study, the relative concentrations of BoNT/A and BoNT/E were selected empirically to produce equivalent levels of response.

2.6. Data analysis

Unless stated otherwise, all values are expressed as

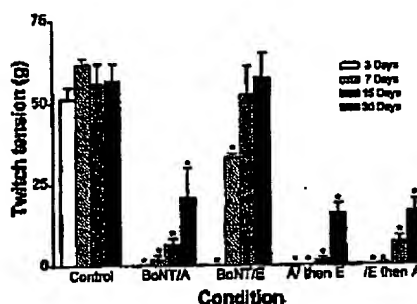


Fig. 2. Twitch tensions recorded in situ from EDL muscles at 3, 7, 15 and 30 days under control conditions or after local i.m. injection of BoNT. Five MU of BoNT/A or 20 MU of BoNT/E were injected into the EDL muscle to produce total paralysis in 48 hr. The left EDL was injected with BoNT, and the right EDL served as control. Sequential injection of BoNT/A followed by BoNT/E (A then E) or BoNT/E followed by BoNT/A (E then A) were performed at 48-h intervals to allow completion of SNAP-25 proteolysis from the first prototype. Each bar represents the mean \pm SE from 3–7 rats. Asterisks indicated values that are significantly different from control ($P < 0.05$).

the mean \pm S.E. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test (GraphPad, InStat[®] ver 3.01, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of BoNT on *in situ* contractions

Control EDL muscles stimulated *in situ* with 0.2 ms supramaximal indirect pulses produced twitch tensions ranging from 51 to 62 g; these tensions were stable during the 30-day period of observation (Fig. 2). Injections of 5 MU of BoNT/A or 20 MU of BoNT/E led to complete paralysis of EDL muscles within 48 hr. At 3, 7, 15 and 30 days after exposure, EDL muscles were tested *in situ* for recovery from BoNT intoxication. At day 3, muscles exposed to either serotype continued to exhibit total paralysis; even 100 Hz repetitive stimulation failed to elicit a detectable response. When examined at day 7, measurable recovery of tension was observed in muscle exposed to either serotype, but the extent of recovery differed markedly. Muscles injected with BoNT/E produced 33.1 g of tension at day 7 (67% of control), whereas those treated with BoNT/A generated only 1.5 g of tension (2% of control). Preparations exposed to BoNT/E continued to recover rapidly, such that by day 15, twitch tensions were restored essentially to control levels. By contrast, tensions in BoNT/A-treated muscles were only 6.4 g at day 15 (11% of control) and 20.6 g at day 30 (36% of control). These recovery rates are in agreement with those reported in previous studies (Sellin et al., 1983; Adler et al., 1996).

3.2. Sequential serotype application

EDL muscles were first injected with 5 MU of BoNT/A; these muscles were completely paralyzed 48 h later, consistent with cleavage of the C-terminal 9 residues from SNAP-25 (Fig. 1). Muscles were subsequently injected with 20 MU of BoNT/E, which is expected to lead to cleavage of an additional 17 residues from P197 to yield the characteristic BoNT/E fragment P180 and two small peptides (Fig. 1). If P197 is responsible for the long duration of BoNT/A toxicity, exposure to BoNT/E will transform P197 to P180 and convert the recovery rate to that appropriate for serotype E. Contrary to this prediction, the data clearly indicated that the recovery rate following sequential exposure to BoNT/A and BoNT/E resembled the profile expected for serotype A rather than that for serotype E (Fig. 2).

Sequential serotype injections were also performed

in reverse order: BoNT/E was administered first followed 48 h later by BoNT/A. Under this condition, BoNT/A should have no effect on SNAP-25, since prior exposure to BoNT/E would have removed the C-terminal 26-mer peptide fragment containing the BoNT/A cleavage site. Accordingly, if the prolonged toxicity observed with BoNT/A is due to persistence of P197, BoNT/A will be incapable of generating this fragment when applied after BoNT/E. However, the data indicate that injection of BoNT/A after BoNT/E leads to sustained paralysis resembling that encountered with BoNT/A alone (Fig. 2). These results, in conjunction with those of the first sequential paradigm, suggest that differences in paralysis times observed with serotypes A and E are determined by the persistence of active BoNT/A rather than by differences in the lifetime of truncated SNAP-25 fragments.

3.3. Effect of BoNT on muscle weight

Examination of EDL muscle weights in rats exposed to sequential injections of BoNT serotypes confirmed the results of tension measurements. Control EDL muscles showed a gradual increase in wet-weight during the 30-day period of observation (Fig. 3), approximately in proportion to increases in body weight. In the same animals, contralateral muscles injected with BoNT/A underwent marked atrophy that was evident as early as 7 days after toxin administration (Duchen, 1971). The loss of weight in BoNT/A-injected EDL muscles was progressive; at 15 and 30 days after toxin injection, muscle weights were reduced to 48 and 37% of control, respectively (Fig. 3). In con-

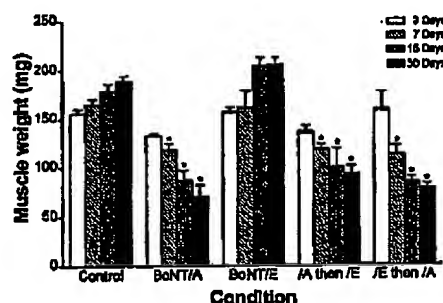


Fig. 3. EDL muscle wet-weights determined at 3, 7, 15 and 30 days under control conditions and after local i.m. injection of BoNT. Muscle weights were obtained after tension recordings. The conditions are identical to those in Fig. 2. Each bar represents the mean \pm SE from 3-7 rats. The left EDL was injected with BoNT, and the right EDL served as control. Asterisks indicated values that are significantly different from control ($P < 0.05$).

trast, EDL muscles in rats injected with BoNT/E showed a moderate increase in wet-weight over the 30-day period of observation. With either sequential serotype injection, however, muscles underwent a reduction in weight similar to that observed in BoNT/A alone (Fig. 3). Thus, exposure to BoNT/A leads to prolonged paralysis and atrophy independent of the presence of BoNT/E.

3.4. Evidence for sequential serotype action

To ensure that the results of the sequential serotype experiments reflect the contribution of both serotypes, it is necessary to demonstrate that each serotype enters the nerve terminal and acts on its respective target site. Action of the first serotype can be inferred by the observed paralysis, but the situation is less apparent for the second serotype. To address this problem, we used the ability of the potassium channel blocker 3,4-DAP to augment tensions selectively in BoNT/A-treated muscles to confirm the presence of the second serotype (Molgo et al., 1980, 1989; Simpson, 1986; Adler et al., 1996). Fig. 4 shows the results of experiments in which 3,4-DAP was applied to muscles exposed to BoNT/A, BoNT/E or BoNT/A followed by BoNT/E. Ex-

posure of a pair of hemidiaphragm muscles to 300 pM BoNT/A led to a gradual depression of twitch tension, culminating in total paralysis in ~90 min. BoNT/E (500 pM) was then added to one of the two BoNT/A-paralyzed muscles and to a control muscle; the latter underwent a rapid depression of twitch tension. After the BoNT/E-exposed muscles became totally paralyzed, excess toxin was removed by washout, and 100 μ M 3,4-DAP was added to all three hemidiaphragm preparations. The muscle exposed to BoNT/A alone showed a prompt and substantial reversal of paralysis, whereas the hemidiaphragms exposed to BoNT/E alone or sequentially to BoNT/A followed by BoNT/E remained paralyzed for the duration of the experiment. These results suggest that BoNT/E can enter BoNT/A-intoxicated nerve terminals, convert P197 to P180, and thus make the muscles refractory to 3,4-DAP action. This conclusion is supported by the finding of cleavage of P197 to P180 in permeabilized chromaffin cells exposed to BoNT/A and BoNT/E (Lawrence et al., 1997). Co-penetration of multiple serotypes in intact muscle, specifically, BoNT/A with BoNT/B or tetanus toxin, has also been demonstrated (Gansel et al., 1987).

Additional evidence in support of the above con-

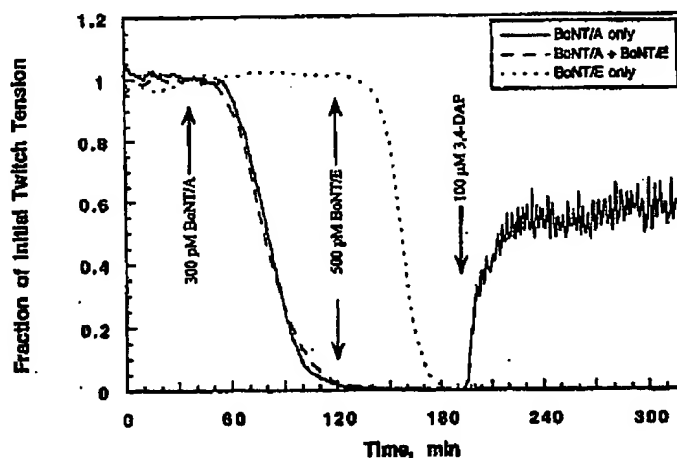


Fig. 4. Twitch tensions recorded *in vitro* in three mouse hemidiaphragm muscles to demonstrate sequential serotype action. At the first arrow, two preparations received 300 pM BoNT/A, and after a 20–30 min lag both muscles underwent complete paralysis. At the second arrow, a control and one of the BoNT/A-treated muscles were exposed to 500 pM BoNT/E. The control diaphragm showed a rapid decline in tension. At the third arrow, all three muscles received 100 μ M 3,4-DAP. The BoNT/A-treated preparation exhibited rapid recovery to ~60% of control, but the preparations incubated in BoNT/E or with BoNT/A followed by BoNT/E showed no recovery by 3,4-DAP. These results suggest that BoNT/E was able to enter the nerve terminal of BoNT/A-treated preparations and cleave P197 to P180. Exposure to BoNT/A and BoNT/E in reverse sequence led to a similar lack of effect of 3,4-DAP (not shown). These data are representative of 4–6 experiments. The time to 50% block was similar for the two BoNT serotypes (47.3 \pm 4.6 min for BoNT/A and 42.9 \pm 3.8 min for BoNT/E).

clusion in intact neurons was obtained by exposing rat cortical cultures to BoNT/A and BoNT/E and monitoring the cleavage state of SNAP-25 by western blot analysis (Fig. 5). Lane 1 shows blots for syntaxin (another presynaptic protein) and SNAP-25 from control cortical neurons. Lane 2 was obtained 24 h after cells were exposed to BoNT/A (50 pM) and demonstrates cleavage of SNAP-25 from 25 to 24 kDa due to loss of the C-terminal 9 residues. The specificity of BoNT/A for SNAP-25 is indicated by the absence of changes in syntaxin (Binz et al., 1994). Incubation of cortical neurons in BoNT/E led to the appearance of a 22 kDa peptide fragment, as expected from the removal of C-terminal 26 residues from SNAP-25 (lane 3). In the final experiment, cells were exposed to BoNT/A followed 24 h later by BoNT/E. After waiting an additional 24 hr for completion of the BoNT/E reaction, cells were probed for syntaxin and SNAP-25 (lane 4). The SNAP-25 cleavage profile in cells exposed sequentially to BoNT/A and BoNT/E resembled that observed in BoNT/E alone. This finding suggests that BoNT/E was able to enter cells after BoNT/A and cleave SNAP-25 at its characteristic site, in spite of the truncation of SNAP-25 from prior exposure to BoNT/A.

4. Discussion

The *in situ* local injection model was used to probe the basis for differences in the recovery rates following exposure of EDL muscles to BoNT/A and BoNT/E. In agreement with observations from systemic botulinum

poisoning, recovery from local BoNT/E exposure was rapid and essentially complete within 15 days after an *i.m.* injection of a paralytic dose (20 MU). Recovery from a paralytic dose of BoNT/A (5 MU) was slow, and reached only 11% of control at day 15 and 36% of control at day 30 (Fig. 2).

In the current study, two limiting cases were considered to account for the marked differences in recovery rates of serotypes A and E. The first assumed that the active toxin has a brief residence time in the nerve terminal and that the observed recovery rates reflect the disappearance of truncated SNAP-25 fragments. The second assumed that recovery from muscle paralysis is limited by the duration of active toxin and that truncated SNAP-25 cleavage products are eliminated more rapidly from the nerve terminal, thus having little influence on the duration of muscle paralysis.

These proposals were tested by injecting paralytic doses of BoNT/A and BoNT/E into the EDL muscle and monitoring recovery over a 30-day period. Sequential injection of BoNT/A followed by BoNT/E 48 h later gives rise initially to P197 from cleavage of intact SNAP-25, followed by conversion of P197 to P180 from action of BoNT/E on the first fragment (Figs. 1 and 5). The reverse injection sequence generates P180 from BoNT/E action, which is expected to remain unchanged following BoNT/A administration. The prediction, according to the first hypothesis, is that muscles injected sequentially by BoNT/A followed by BoNT/E should recover at the same rate as those injected with BoNT/E alone, with a possible lag of 48 h corresponding to the time that P197 was present. Muscles injected sequentially with BoNT/E followed

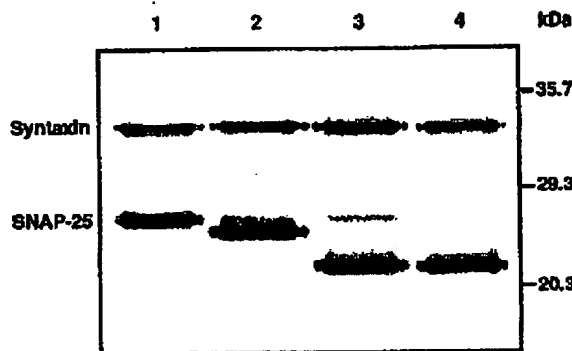


Fig. 5. Western blot of cultured rat cortical neurons treated with BoNT/A, BoNT/E or both neurotoxins. Primary cortical cultures were incubated with 50 pM BoNT/A (lane 2), 90 pM BoNT/E (lane 3) or 50 pM BoNT/A followed 24 h later by 90 pM BoNT/E (lane 4). Control cells with intact SNAP-25 are shown in lane 1. Cells were collected by brief trypsinization, centrifuged, dissolved in 1% SDS/0.1% β -mercaptoethanol and boiled for 5 min. Syntaxin, a presynaptic membrane protein that is not cleaved by BoNT/A or BoNT/E, appears unchanged in all four samples. Note that BoNT/E was able to cleave both intact and BoNT/A-truncated SNAP-25.

by BoNT/A should also recover at the same rate as those injected with BoNT/E, since the cleavage site for BoNT/A has already been removed by prior action of serotype E (Fig. 1). Contrary to these expectations, recovery rates with either injection sequence resembled the protracted rate observed with BoNT/A alone, rather than the more rapid rate expected for BoNT/E. These results make it unlikely that the truncated SNAP-25 fragment generated by BoNT/A action determines the time course of recovery and support, the second limiting case, which attributes the longer duration of BoNT/A-mediated paralysis to a more sustained intracellular activity of BoNT/A light chain.

Using an alternative approach for toxin administration, Eleopra et al. (1998) found that co-injection of BoNT/A and BoNT/E in human extensor digitorum brevis muscle led to a time course of paralysis similar to that of BoNT/E, rather than the much longer duration characteristic of BoNT/A action. In essence, simultaneously applied BoNT/E shortened the duration of BoNT/A-induced muscle paralysis. The authors concluded that the duration of paralysis is determined by the lifetime of the truncated BoNT/A-generated SNAP-25 fragment, rather than by the persistence of active toxin that we have proposed in this study.

It is unclear whether the discrepancy between the findings of the present study and those of Eleopra et al. (1998) represents a fundamental difference between rodent and human muscle, or reflects differences between simultaneous and sequential BoNT administration. With regard to the first suggestion, it is possible that rodents can degrade BoNT/A-truncated SNAP-25 more rapidly than humans. Thus, in the former, toxin activity may be rate limiting for recovery, while in the latter, recovery may be limited by the removal of truncated SNAP-25 cleavage products. One can obtain a reasonable approximation for the rate of disappearance of the BoNT/A-truncated SNAP-25 from the recent study of Keller et al. (1999). These authors showed that the steady-state ratio of intact and BoNT/A-truncated SNAP-25 did not change appreciably with time for up to 72 days after an initial exposure to BoNT/A. Since synthesis and transport of SNAP-25 is rapid (Loewy et al., 1991), the finding that BoNT/A-truncated SNAP-25 did not accumulate in cells with persistent BoNT/A activity suggests that this fragment must be degraded as rapidly as its formed. In the optic tract and superior colliculus, elimination of control SNAP-25 was found to have an approximate half-time of 5 days (Loewy et al., 1991). This is similar to the apparent half-time for disappearance of BoNT/E-truncated SNAP-25 estimated by Keller et al. (1999). From the above, it would not be unreasonable to expect a similar half-time for removal of BoNT/A-truncated SNAP-25 from the rat EDL muscle. Elimination of P197 thus appears to be too rapid to account

for the time-course of recovery of muscle contractility (Fig. 2). In this context, de Paiva et al. (1999) demonstrated that initial recovery of muscle function after BoNT/A exposure arises from the activity of newly formed sprouts, whereas >3 months are required for complete restoration of function in the original nerve terminal. Thus, recovery of normal nerve terminal function in muscles exposed to BoNT/A is even slower than would be indicated by the return of contractility. Although data in humans are not available on either the persistence of BoNT/A enzymatic activity or on the degradation rate of BoNT/A-truncated SNAP-25, it is possible that elimination of the latter is rate limiting in human muscle, and conversion to P180 would then accelerate recovery of muscle function.

According to the second possibility, simultaneously applied toxin may give results that differ from those obtained following sequential administration due to possible competition of BoNT/A and BoNT/E for factors such as cell surface receptors. A number of candidates have been suggested as protein receptors for clostridial neurotoxins (Nishiki et al., 1994; Schengrund et al., 1996), although many of these may be 'nonproductive' (Daniels-Holgate and Dolly, 1996). Li and Singh (1998) have reported that synaptotagmin I serves as the protein component of the cell surface receptor for both BoNT/A and BoNT/E. With simultaneous application of serotypes A and E, competition for a common receptor could result in a reduced binding of each serotype (Li and Singh, 1998). Under this condition, recovery from paralysis may exhibit a time course that is no longer dominated by BoNT/A. In addition, simultaneous injection of BoNT/A and BoNT/E may also lead to competition for internalization sites as well as for access to substrate. The consequences of serotype competition for binding, endocytosis and substrate availability would appear to be more pronounced with the subparalytic doses of toxin, such as that employed in the human study (Eleopra et al., 1998). Sequential serotype administration avoids these difficulties, since the toxin binding and internalization processes do not overlap in time, and was therefore preferred in the present investigation. Although differences in the mode of administration or in species may contribute to the differences between our findings and those of Eleopra et al. (1998), final resolution of this issue must await further investigation.

A number of studies have addressed the interactions of SNAP-25 with other proteins in the regulation of neurotransmitter release. Two of these proteins, synaptotagmin, located at the active zone of the nerve terminal, and synaptobrevin, associated with synaptic vesicles, form a ternary complex with SNAP-25. Formation of this complex is required for the docking and subsequent fusion of synaptic vesicles within active zones in preparation for neurotransmitter release (Pevsner et

al., 1994; Fasshauer et al., 1998). The rationale for considering P197 to be inhibitory came from findings that this fragment retains its ability to interact with syntaxin and synaptobrevin in vitro (Hayashi et al., 1994, 1995). To account for the finding that transmitter release is inhibited in the presence of P197, it may be assumed that it forms an inactive ternary complex with synaptobrevin and syntaxin that is incapable of initiating synaptic vesicle fusion (Raciborska and Charlton, 1999; Washbourne et al., 1999). In support of this suggestion, recombinant P197 was shown to inhibit insulin secretion in pancreatic HIT-T15 cells (Huang et al., 1998), providing direct evidence that appropriate concentrations of P197 can inhibit the release process. Since conversion of P197 to P180 did not accelerate recovery of tension in the present study, removal of P197 was presumably not rate limiting for recovery of muscle tension (Fig. 2). Huang et al. (1998), in fact, suggested that P197 is rapidly degraded in cultured HIT-T15 cells.

The ability of noncleavable SNAP-25 mutants but not wild-type SNAP-25 to restore secretion in BoNT/A-inhibited chromaffin cells further indicates the primary role of active toxin in determining the duration of BoNT/A action (O'Sullivan et al., 1999). The rescue of transmitter release by noncleavable SNAP-25 mutants also demonstrates that P197 can be rapidly displaced from the SNARE complex. Accordingly, native SNAP-25 from de novo synthesis should also be capable of displacing BoNT/A-truncated SNAP-25 to re-establish function if it were not for continued toxin-mediated proteolytic activity.

The dominance of catalytically-active BoNT/A in determining the duration of BoNT/A intoxication is supported by two additional lines of evidence. First, inhibition of catecholamine release can be rapidly reversed in BoNT/A-intoxicated chromaffin cells by administration of BoNT/A light chain antibodies (Bartels et al., 1994). This finding indicates that persistence of BoNT/A catalytic activity is the major factor underlying continued toxin-mediated impairment of the release process. Second, a 7-day osmotic minipump infusion of 3,4-DAP antagonized BoNT/A-induced paralysis in rat EDL muscle (Adler et al., 2000). However, 3,4-DAP-augmented tensions were not maintained after removal of minipumps but returned to values similar to those observed in muscles exposed to BoNT/A alone. Based on the reported turnover rates for SNAP-25 (Loewy et al., 1991; Lane and Liu, 1997; Sanders et al., 1998), the 7-day treatment with 3,4-DAP and the consequent muscle activity should have led to a substantial replacement of BoNT/A-truncated-SNAP-25 by intact SNAP-25 and thus to a sustained improvement of muscle tension were it not for continued BoNT/A enzymatic activity.

In addition to the large truncated SNAP-25 frag-

ments, BoNT-mediated protease activity also generates small peptides that are released following cleavage of SNAP-25 (Fig. 1). Action of BoNT/A gives rise to P9, whereas proteolysis by BoNT/E generates P26. An additional small peptide (P17) is generated during sequential application of BoNT/A and BoNT/E, but no additional small peptides are likely to form when BoNT/E is followed by BoNT/A, since the P26 peptide is not readily cleaved by BoNT/A (Vaidyanathan et al., 1999). These smaller fragments have also been found to interfere with the fusion and release of synaptic vesicles. Synthetic peptides homologous to 20 and 26 C-terminal residues of SNAP-25 have been shown to inhibit catecholamine release from adrenal chromaffin cells, block the formation of the ternary complex and depress the release of acetylcholine from *Aplysia californica* buccal ganglia (Gutierrez et al., 1995, 1997; Ferrer-Montiel et al., 1998). Gutierrez et al. (1997) suggested that the inhibitory actions of BoNT/A and BoNT/E are derived from both, the loss of intact SNAP-25 and the accumulation of the small SNAP-25 fragments. These findings imply that P26 is degraded rapidly relative to P9 to account for the shorter duration of BoNT/E intoxication. As with the large truncated SNAP-25 fragments, however, it still remains to be demonstrated that inhibitory concentrations of the smaller fragments are actually generated during in vivo BoNT activity.

Assuming that the duration of paralysis is determined by the lifetime of active toxin, it is not obvious why one of the two serotypes should have such a protracted intracellular lifetime. One possibility is that BoNT/A is selectively stabilized by phosphorylation. In support of this proposal, Ferrer-Montiel et al. (1996) demonstrated that tyrosine phosphorylation of BoNT/A increases both, its intracellular stability as well as its catalytic activity. However, phosphorylation is not unique to BoNT/A; more recent studies have demonstrated a similar stabilization and enhanced enzymatic activity for BoNT/E (Encinar et al., 1998). Thus, if phosphorylation is the critical factor imparting stability to BoNT/A, then BoNT/E must be subjected to an environment inhospitable to this modification in vivo.

An additional possibility for the unusually long survival of BoNT/A may be that it contains signal sequences that could allow for association with endogenous proteins or organelles, thus protecting BoNT/A from rapid degradation. This possibility can be tested by use of a recombinant BoNT/A where putative signal sequences are modified by site-directed mutagenesis. Alteration of such sequences should destabilize BoNT/A so that its intracellular lifetime may be reduced to resemble that of shorter acting clostridial neurotoxins.

In summary, our findings indicate that long-term

paralysis from BoNT/A intoxication in rat EDL muscle is determined by persistence of toxin activity and that neither prior nor subsequent exposure to BoNT/E alters the observed recovery time.

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